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A REVIEW OF THE GENETICS OF ALCOHOLISM AND A CONFIRMATORY STUDY OF AN ACUTE ALCOHOL WITHDRAWAL QUANTITATIVE TRAIT LOCUS IN MICE

1998, 83 Pages, Master of Science in Genetics, The Pennsylvania State University

Alcoholism is a costly human disease both fiscally and emotionally. Therefore, a greater understanding of the disease could potentially save money and lives. The past 20 years of research have provided substantial evidence that alcoholism is, at least in part, hereditary. The specific genes responsible for various other diseases have been discovered in the past decade, but alcoholism is too complex to offer such a simple solution. Although certain genes might help to predict the disease, it is a complex, multigenic trait for which "the gene" will never be discovered.

In order to understand the genetics of this complex trait, we can exploit the fairly recently developed method of quantitative trait loci (QTL) mapping. Furthermore, an application of the QTL method to an animal model of alcohol withdrawal should be one of the most rewarding realms to explore. Animal models of an alcoholism component have proven valid and useful in understanding withdrawal, one of an assortment of various presumed components of alcoholism.

A number of QTLs for acute alcohol withdrawal has been nominated through recombinant inbred methodology, but these nominees require replication through a number of methods. One of the most efficient methods is genotypic selection, in which animals are made homozygous for a single QTL in one generation, while the rest of the genotype segregates. This study uses genotypic selection to confirm a QTL for acute alcohol withdrawal on mouse Chromosome 4.

The Pennsylvania State University

The Graduate School

Intercollege Graduate Program in Genetics

A REVIEW OF THE GENETICS OF ALCOHOLISM AND A CONFIRMATORY STUDY OF AN ACUTE ALCOHOL WITHDRAWAL QUANTITATIVE TRAIT LOCUS IN MICE

A Thesis in

Genetics

by

Jeffrey T. Geraghty

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of the Requirements
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ABSTRACT

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Chapter 1

LITERATURE REVIEW OF THE GENETIC BASIS OF ALCOHOLISM

Background

Alcoholism is a devastating and costly human disease. In the past 20 years, new technology and techniques have opened the door to a greater understanding of the complex disease. Researchers are convinced that a predisposition to developing alcoholism is heritable. While studies of specific genes reveal little about the hereditary nature of alcoholism, complex genetics techniques promise more. Alcohol withdrawal, a component of alcoholism, provides a simulacrum that can be studied in animals. The application of quantitative trait loci (QTL) techniques to mouse models of alcohol withdrawal could someday lead to better diagnostics and therapeutics for alcoholism.

Alcoholism : The Disease

The Cost

Some 10% of adults who consume alcohol are considered alcoholics or experience drinking problems to some degree. The Epidemiological Catchment Area

(ECA) study found that 16% of the general population experienced alcoholism at some point during their lifetime (Helzer & Burnam, 1991). Furthermore, as cause for greater concern, alcohol use is increasing in many countries worldwide, including the United States and the former Soviet Socialist Republic (Bissel 1997). In 1993, as many as 16.2% of males ages 18 to 25 reported having partaken in "heavy drinking"* on five or more days in the previous month (Maisto, Galizio, & Connors, 1995). In addition, alcohol creates a fiscal sink, as approximately $136 billion per year is spent in dealing with the social and health problems arising from its use (Weinrieb & O'Brien, 1997). Finally, alcohol consumption and its secondary effects cause more than 100,000 deaths per year in the United States. Alcohol holds exclusive claim to 18% of these deaths (Grant et al., 1994).

Prior to the 1940's, alcoholics were understood to lack moral will, rather than be victims of a disease. Various speeches and publications of the period testify to this sentiment. For example, a man by the name of J.E. Todd spoke to the General Association at Middletown of the lack of morality inherent in alcoholics: "Every human soul is worth saving, but what I mean is, that if a choice is to be made, drunkards are about the last class to be taken hold of...(Todd, 1882)." A transcript of his speech was later published and distributed to churches throughout the area. Alcoholism came to be regarded as a disease tens of years later (Jellinek, 1960).

With the advent of the "disease concept" of alcoholism, clinicians and researchers alike have striven to demystify its causal agents. Particularly, they have tried to answer

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* Heavy drinking in this context is defined as having five or more drinks per occasion.

the question: "To what extent does an individual's genetic constitution contribute to the disease?" Alcoholism's complex nature makes the question difficult to answer.

The Complexity

Alcoholism is a complex human disorder that is caused and maintained by a combination of biological, psychological, and sociological factors (Maisto, Galizio, & Connors, 1995). This nexus of causality combines with an assortment of definitions and classifications to create a heterogeneous phenotype called alcoholism. Researchers claim that the "necessary or defining attribute for alcoholism has yet to be specified (Tarter & Vanyukov, 1994)." Furthermore, consensus regarding the meaning of this condition is evasive.

Various researchers have broken the domain of alcohol abuse into various components in an attempt to understand it. It is divided into two parts in the American Psychiatric Association's *Diagnostic and statistical manual*, fourth edition: alcohol dependence and alcohol abuse. Dependence upon alcohol is indicated by symptoms of tolerance or withdrawal. Alcohol withdrawal is evidenced by symptoms that manifest themselves approximately 12 hours following heavy, prolonged alcohol ingestion (DSM-IV, 1994).

For convenience, we define alcohol dependence as consisting of two components: physical dependence and psychological dependence. Physical dependence, the physiological requirement for alcohol, can be deduced from withdrawal symptoms that are alleviated upon administration of the drug. Psychological dependence, the perceived

need for alcohol, can be inferred from verbal reports as well as from alcohol-seeking behavior (Crabbe, McSwigan, & Belknap, 1985).

Physical dependence cannot be directly assessed but rather only inferred from the severity and duration of the withdrawal syndrome. Symptoms of alcohol withdrawal can be classified into three phases, as seen in Table 1-1. Human hangovers represent a minor withdrawal syndrome, because they are the body's readjustment to a non-alcohol condition (Maisto, Galizio, & Connors, 1995). These symptoms vary widely, and the blood alcohol level need not have fallen to zero for an individual to exhibit alcohol withdrawal. For example, in their book *Diagnostic Criteria for Alcoholism and Alcohol Abuse*, Mendelson & Mello (1985) report that withdrawal states can occur when blood ethanol levels are still relatively high. Dora Goldstein (1972) observed that mice could "surprisingly" be simultaneously intoxicated and in the early stages of withdrawal.

Table 1-1: Three Phases of Alcohol Withdrawal Syndrome

Phase	Onset	Symptoms
1.	As soon as a few hours after drinking has stopped. BAC's can still be above 0.	Tremulousness Perspiration Weakness Drug-seeking behavior
2.	Within about 24 hours of drinking cessation.	Grand mal seizures.
3.	About 30 hours or longer after drinking cessation. Most protracted phase, may last 2-4 days. Often called delirium tremens (DTs).	Severe agitation Confusion Disorientation Continual activity Hallucinations (most often felt as bugs or little animals crawling on the skin) Hallucinations are accompanied by delusions, with high potential for violent behavior without medical management.

Source: Maisto, Galizio, & Connors, 1995

The Heredity of Alcoholism

Some people refrain entirely from consumption of alcohol. Many individuals consume it without ever becoming alcoholics. The remainder of individuals develop, to some degree, alcohol dependence. Since such large individual differences characterize alcoholism, we can conclude that mere experience with alcohol is not enough to induce and sustain alcohol dependence. Instead, factors intrinsic to individuals must account for some of the variation in levels of alcohol use and abuse.

Alcoholism has been known to "run in families" for years. A more recent statistical compilation quantifies that statement. If an individual's father is a clinically diagnosed alcoholic the risk rises to 26% (from about 3 to 4% in the general population) that that individual will become an alcoholic. An alcoholic mother presents only a 2% risk increase. Similarly, an alcoholic brother equates to a 21% risk, while an alcoholic sister only 0.9% (Ehrman & Parsons, 1981).

Studies such as the preceding example that have been performed over the last 20 years soundly support the hypothesis that some humans suffer from a genetic predisposition to develop alcoholism (Crabbe, McSwigan, & Belknap, 1985). Interestingly, Crabbe's conclusion arose from behind a cluttered horizon of various diagnostic criteria. These various diagnostic systems and criteria confounded his study and others like it. Nevertheless, he found a genetic contribution to the disease.

Taking the observations further, Ehrman and Parsons, in the book *Behavior Genetics and Evolution* (1981), confidently state that "conditions of rearing are apparently less influential in producing or deflecting from alcoholism than is

inheritance." Results from twin studies "support the hypothesis that there is a genetic explanation for the development of alcoholism (Bohman, 1978)." Cloninger, Bohman, & Sigvardsson (1981) conclude that susceptibility to alcoholism is heritable.

Likewise, particular components of alcoholism, such as susceptibility to alcohol withdrawal, are now known to be significantly genetically determined. For instance, heritability estimates ranging from 28% (Crabbe et al., 1985) to 33% (Tarantino, McClearn, & Plomin, 1997) describe that proportion of the phenotypic variance of alcohol withdrawal severity in mice that is determined by genetics.

Nevertheless, there exists a paucity of information regarding specific genetic determinants of the disease (Ehrman & Parsons, 1981). It is the nature of the methods used, and not the nature of the disease itself, that is responsible for the dearth of evidence that specific risk genes exist (Devor, 1994). Those few genes that have been implicated as predictors of alcoholism will be discussed next.

Monogenic Trait Inheritance

In the past 15 years, linkage analysis has helped to uncover the genes responsible for a variety of monogenic diseases, such as Huntington's disease (Gusella et al., 1983), cystic fibrosis (White et al., 1985), Duchenne muscular dystrophy (Davies et al., 1983), and others (Risch, 1990). Accorded such knowledge, even the knowledgeable citizen might be waiting for the discovery of the gene that causes alcoholism. Alcoholism is not

such a simple condition, but some alcohol metabolism- or neurotransmission-related genes could have a pronounced effect on individuals.

Application of a classic linkage analysis is inappropriate for finding such genes for alcoholism, because a reasonably accurate genetic transmission model for alcoholism, as for most other complex disorders, is difficult to achieve (Devor, 1994). Therefore, monogenic research has been aimed at understanding genes that affect the metabolic pathway with which alcohol is involved.

Some recent research has been aimed at testing whether alcohol acts at specific receptors such as the γ-aminobutyric acid (GABA)-benzodiazepine receptors. The results of the research are unclear at this point (Maisto, Galizio, & Connors, 1995). However, behavioral studies have implicated decreased GABA-mediated neurotransmissions in creating symptoms of alcohol withdrawal. Though genes involved in the changes of synaptic actions in GABA receptors are not known, recent research has linked chromosomal regions containing genes responsible for GABA synthesis to alcohol withdrawal (Buck et al., 1997).

A few other genes have been implicated as markers of alcoholism in the past 10 years. In 1990 a publication by Blum et al. (1990) announced that one allele of an RFLP in the dopamine receptor D2 (DRD2) region on Chromosome 11q was significantly associated with severe alcoholism. Dopamine is a monoamine neurotransmitter involved with the rewarding effects of alcohol and other drugs (Welsh, 1995). Although results of replicative testing have been mixed, a compilation by Uhl et al. (1993) concludes that the positive evidence outweighs the negative. Overall, the average prevalence of the A1

allele of the Taq1 DRD2 RFLP in alcoholics is 40.5% (n=898) compared to 29.1% among controls (n=748). Such a meta-analysis in the presence of mixed results from individual studies leads to the conclusion that the DRD2 locus plays some role in influencing the severity of alcoholism. Interestingly, DRD2 is also implicated in the severity modification of other neuropsychiatric diseases, such as Tourette's syndrome (Devor, 1994). This evidence implies that this gene plays a general role in interacting with neuropsychiatric disease-specific risk genes.

A second possible marker of alcoholism that has withstood the test of replication is decreased monoamine oxidase-B (MAOB) activity. MAOB is an enzyme involved in the breakdown of monoamine neurotransmitters such as dopamine and serotonin (Anthenelli & Tabakoff, 1995). Monoamine neurotransmitters have been implicated in a number of phenomena related to alcoholism, including a preference for alcohol consumption and a susceptibility to alcohol's rewarding effects (Tabakoff & Hoffman, 1992).

Evidence from various studies suggests that particular subtypes of alcoholics are associated with lower MAOB activities than other subtypes. Studies involving the Devor-Cloninger Type I/II alcoholism dichotomy have found that the more severe Type II alcoholics have lower MAOB activity than do Type I alcoholics (Devor et al., 1993). Similarly to DRD2, decreased MAOB activity has been independently linked to other neuropsychiatric diseases such as neurotic depression and childhood attention deficit disorder (Dowson, 1987).

Another category of genes nominated includes those involved in the oxidative metabolic pathway of alcoholism. These include the genes whose products are the alcohol dehydrogenases (ADH), the aldehyde dehydrogenases (ALDH), and formaldehyde dehydrogenase (FDH). One alternative enzyme form of an ALDH, called ALDH2, may serve to reduce the risk (Devor, 1994).

A mutation in the ALDH2 gene, in which the amino acid residue at position 487 is a lysine rather than a glycine (termed ALDH2*2, as compared to the normal ALDH2*1), leads to a loss of ALDH2 function in the liver. The result is a slow removal of acetaldehyde from the liver, and henceforth the well-known reaction characterized by facial flushing, nausea, palpitations, and lightheadedness (Wolff, 1972).

The frequency of ALDH2*2 is about 50% in the Japanese population, whereas it is in only 2% of Japanese alcoholics. Since a significantly lower proportion of alcoholics have the mutant ALDH2 form that results in loss of function (Harada et al., 1982), it is hypothesized that the reaction produced from loss of ALDH2 function serves as a protective factor against alcoholism (Devor, 1994).

Finally, adenylate cyclase (AC), a membrane-bound enzyme that catalyzes the conversion of ATP to cyclic-AMP, has recently been implicated as a biological-genetic marker of alcoholism propensity. Cells (including neurons) use the enzyme AC to relay signals from a cell's exterior to its interior. The signals can be excitatory or inhibitory (Anthenelli & Tabakoff, 1995). Alcoholics exhibited a significant decrease in AC levels compared with controls. Furthermore, this decreased activity persisted in alcoholics even

after four years of abstinence, meaning that alcoholics might be much more susceptible to some of alcohol's general effects (Tabakoff et al., 1988).

This metabolic-pathway approach has met reasonable success in relation to alcoholism. In addition, even linkage analysis studies into psychological and psychiatric abnormalities show some promising results, for example in dyslexia (Smith et al., 1990). Nevertheless, a caution has been posted regarding the exclusive reliance on monogenic methods in uncovering the etiology of complex diseases (Risch, 1990). Therefore, we must turn to methods designed for the understanding of complex traits.

Complex Trait Genetics

A complex trait is defined as "a trait in which a one-to-one relationship between genotype and phenotype does not exist (Darvasi, 1998)." Alcoholism is a complex trait. Alcoholism is not a unitary disease. It is a complex, heterogeneous, multifactorial disorder involving a plexus of genes and environmental factors, the influence of which ebb and flow over the course of time (Devor, 1994).

Aside from this general complexity, alcoholism is the label for conditions exhibited by an individual when his or her liability phenotype passes a particular severity threshold. The liability phenotype is consistent with a multifactorial model of inheritance (Tarter, 1994).

Among the levels in this multifactorial model are primary risk genes, primary gene-gene interactions, and secondary gene-gene interactions. The primary risk genes include a finite number of alcoholism-specific genes that segregate independently in the

human population, each conferring to the individual a specific increase in the risk of developing alcoholism (Devor, 1994). No alcoholism specific genes have yet been found, and due to the highly pleiotropic nature of genes it is possible that such "primary risk genes" never will be found.

If any two or more of these primary risk genes are present in an individual, the possibility for primary gene-gene interactions exists. The effects of such interactions could range from simple additive (no interaction) risk increase (in which the risks associated with each primary gene are summed linearly), to epistatic effects (in which the primary genes exhibit a synergistic increase in risk of developing alcoholism) (Devor, 1994). Results from our laboratory currently elucidate one example in which a non-linear combination of QTLs occurs in mice (Tarantino, 1998).

Similarly, secondary gene-gene interactions involve an interaction between primary risk genes and other genes. At this level, however, the genes with which the primary risk genes interact are not alcoholism specific. These genes, a part of the developmental milieu in which alcoholism develops, may interact with all primary risk genes in a common manner, or may interact only with some. Moreover, these genes could either increase or decrease the liability towards alcoholism. DRD2, MAOB related genes, and ALDH2 currently reside in this class of genes (Devor, 1994).

Since relatively few distinct alcoholic subtypes exist (Babor et al., 1992), one or both of the following possibilities might be true: a) the levels of interaction are relatively small, or b) the number of risk genes are relatively small and thus manageable (Devor, 1994). If neither of these possibilities were the case in alcoholism, it is hypothesized that

the complex interactions among the levels and/or the genes would create an array of distinct subtypes. Goldman (1993) reported that two or *possibly* more subtypes may be influenced by different patterns of inheritance. Anthenelli & Tabakoff (1995) described the three most common subtype schemes: primary v. secondary, Type I vs. Type II, and Type A vs. Type B. The latter two involve differences in severity, while the former depends on whether the alcoholic has previously exhibited anti-social personality disorder (ASPD). The low number of *distinct* subtypes contains the promise that an analysis of this complex, quantitative trait called liability for developing alcoholism will yield practical results.

It is important to keep in mind that there might be a large number of distinct subtypes of alcoholism yet to be discovered. Such a case would be similar to mental diseases in the early 1900's, when almost all retardation was lumped together. Research has since uncovered an array of distinct subtypes in mental disease, including Down's Syndrome and Phenylketonuria (PKU). Perhaps fifty years from now, a number of distinct subtypes of alcoholism will surface.

Until recently, the molecular genetic analysis of a complex trait like alcoholism has been virtually impossible due to a lack of molecular and analytical tools. However, researchers have more recently succeeded in detecting and mapping loci responsible for variation in certain complex traits. Complex phenotypes can now be assessed quantitatively. Quantitative trait loci (QTLs) are locations in the genome that contain a gene or genes that account for an appreciable portion of the variability of a complex trait.

(Gelderman, 1975). The utility of the QTL method in understanding complex phenotypes is the topic of the next chapter in this report.

Chapter 2

THE QTL METHOD AS A MEANS FOR UNDERSTANDING THE GENETICS OF ALCOHOLISM

The Utility of the QTL Method

The proposition that genes are of only massive Mendelian effect or of miniscule multigene effect is counterintuitive. Rather, evidence exists that there is probably a continuum of gene effect size (McClearn et al., 1991). Furthermore, a gene might have different effects against different residual genotypes, or genetic backgrounds (Hummel, Coleman, & Lane, 1972).

McClearn et al. (1991) points out that "Recent developments in molecular genetics have provided tools for exploring the middle ground of genetic influence: genes whose effects are not overwhelming, but which account for appreciable proportions of the variance." These tools have paved the way for QTL research. QTL research allows investigators to highlight regions of the genome which account for an appreciable portion of the genetic variance of a trait like alcoholism. Thanks to the QTL method, researchers can now uncover the previously shrouded genes of medium effect size, of which alcoholism genes are almost certainly a part.

The use of Mendelian methods to understand individual genes' roles in complex phenotypes faltered before the advent of QTLs. Any genetic analysis of complex

phenotypes relied upon some unrealistic assumptions. These assumptions were that 1) gene frequencies at all loci are approximately equal, 2) the genes effects and dominance relations are more or less the same, and in some contexts 3) an indefinitely large number of genes affect the trait (Falconer & Mackay, 1996). On the contrary, Michel Georges (1997) makes an arguable point that numerous independent confirmation studies make it clear that most quantitative traits involve only a limited number of genes, each with significant effect.

Recently the advent of QTL techniques has presented a means by which to understand individual genes. Using QTL techniques it is possible to discover genes with less of an effect than the typical Mendelian effect of 3 standard deviations from the population mean (σ_P). Effects of 0.5 – 1.0 σ_P are large enough for QTL detection.

For example, an allele for an additive trait with a standardized effect of 2a=1 σ_P (two alleles summed) at a frequency of p = q = 0.5 will account for 12.5% of the total phenotypic variance by the equation:

$$V_A = 2pqa^2 \qquad [2.1]$$

Without the broad-sweeping QTL approach, a gene of such effect could not be detected behind the background variance created by the residual genotype (Falconer & Mackay, 1996).

There exist several methods by which to detect more influential genes, including multimodal distribution, backcrossing with selection, non-normal distribution, heterogeneity of variance, offspring-parent resemblance, and complex segregation analysis. Inferring the presence of a major gene using the preceding methods still does

not lead to a conclusion as to what these genes are. Furthermore, the loci contributing to the polygenic fraction of the variation remain undiscovered, contrary to the case of using a QTL approach.

Therefore, current QTL research is aimed at identifying all loci contributing to variation in a quantitative trait, from the largest to the smallest (within the limits of experimental resolution). In addition, this research aims to place these QTLs on a chromosomal map, and subsequently clone the genes responsible.

QTL Technology

The technology of current QTL methods extend single gene mapping techniques, which are based on linkage disequilibrium between alleles at a marker locus and alleles at the linked gene. Therefore, researchers must meet one major requirement before commencing QTL analysis: they must have access to a linkage map of polymorphic marker loci covering the species entire genome.

The required markers should be highly polymorphic to allow distinction between the strains or lines. They should also be neutral markers with regard to reproductive fitness. The unfolding discovery of DNA-based markers--including variable number tandem repeats (VNTRs, also called minisatellites) , microsatellites, and restriction fragment length polymorphisms (RFLPs)--and the development of the polymerase chain reaction (PCR) have released QTL analysis from concern over the otherwise formidable preceding requirements.

Prior to the widespread application of PCR and the discovery of DNA-based markers, researchers utilized cryptic protein variation such as blood group antigens and electrophoretically distinguishable enzyme alleles in their quest to understand genes of limited effect size. Such tools met the requirement of neutrality, but were helplessly few and only mildly polymorphic.

DNA-based markers, on the other hand, successfully fill each requirement. Microsatellite markers, for example, are segments of DNA containing small arrays of simple tandem repeats of varying lengths. These markers are interspersed throughout the genome. One strain of animal might have 10 repeats at a particular marker, while the contrasting strain contains 12. Such small differences are detectable through the polymerase chain reaction, which amplifies such a small region hundreds of millions of times , so that the polymorphism can be detected through gel electrophoresis (Falconer & Mackay, 1996).

Likewise, VNTRs are defined in *Human Molecular Genetics* as moderate sized arrays of tandem repeats dispersed throughout considerable portions of the nuclear genome (Strachan & Read, 1996), and RFLPs are intraspecific variations occurring in the length of DNA fragment produced through digestion by a specific endonuclease (King & Stansfield, 1997). McClearn et al. (1991) note that "The potential for this associationistic approach was amplified enormously by the discovery of restriction fragment length polymorphisms (RFLPs) a decade ago (Botstein et al., 1980)." In 1994 Copeland et al. reported that more than 3500 microsattelite markers and 1300 RFLPs had been identified

and mapped in the mouse. Today, more than 6331 such markers have been mapped in the mouse (Genetic and physical maps of the mouse genome, 1998).

QTL Detection

In conjunction with the preceding technology, two general methods exist for detecting and mapping QTLs. The first involves crosses between animals that differ for the trait of interest, and the second involves segregating populations. Since this research deals with QTLs nominated by the former method, the discussion will be so restricted.

Crosses between animals differing for a trait are the most effective means of discovering QTLs when the lines are fixed for alternate alleles at both the QTL and the marker loci. This fixation ensures maximum linkage disequilibrium between loci in the F_1. The alleles should exist in association, meaning that those alleles which increase the trait should be homozygous in one parental line, whereas alleles which decrease the trait should be homozygous in the other parental line.

In contrast to association, alleles commonly exist in dispersion, meaning that each parental line is fixed for some increasing alleles and some decreasing alleles. Under such conditions, it is possible to locate QTLs, but with less power.

A detection of QTLs by the above method relies upon a relatively simple paradigm: individuals are scored for the continuous phenotype, and genotyped for markers throughout the genome. A QTL is located at markers for which there is a significant difference between mean phenotypes within genotypic marker classes.

A marker association indicates one of two possibilities: 1) the marker is the gene affecting the trait, or 2) the marker is near (linked to) the gene that affects the trait. A high association denotes a gene of significant effect in close proximity to the marker. A low association, however, could mean either a nearby gene of moderate effect, or a more distant gene of substantial effect (McClearn et al., 1991).

The use of recombinant inbred (RI) strains in animal models will be a most important tool in the process of detecting QTLs for certain aspects of alcoholism (McClearn et al., 1991). RI strains result from the full inbreeding of the descendants of an F_2 cross between 2 standard inbred strains. Such a strategy redistributes the F_2 genetic variance so that it exists almost entirely between strains, and is nearly absent within strains (Belknap et al., 1993)

Recombinant inbred methodology can be applied to the search for QTLs by a straightforward extension of linkage to association analysis. Using the Pearson product-moment correlation (the maximum likelihood estimator of the coefficient of correlation (Neter et al., 1996)) across RI strains between a marker and phenotypic scores for a quantitative trait, it is possible to arrive at the same test of significance as a t-test of mean differences between the strains. This correlation can then be assessed for each polymorphic locus in the RI series for which chromosomal location is known. The emergent pattern highlights chromosomal regions with potential QTLs, additionally contributing an estimate of their effect size (McClearn et al., 1991).

Alcoholism QTL research has already exploited the previously mentioned RI method. Oliverio and Eleftheriou (1976) suggested a locus with an effect on the

influence of alcohol administered on activity level. Subsequently, Crabbe et al. (1983) promulgated a more typical multgenic model for effects of administered alcohol on open field activity, while showing evidence of major gene effects in alcohol acceptance and severity of withdrawal syndromes.

Additional QTL Characteristics

Recent research has elucidated a number of aspects of QTLs that merit mentioning. First, the number of QTLs detected is always an underestimate of the true number of QTLs in the genome for the following reasons. Since the resolution of a typical detection experiment is roughly 20 centiMorgans (cM), two closely linked QTLs would appear as one. Furthermore, multiple QTLs that are tightly linked might influence the trait in different directions, thus canceling out each other's effects and avoiding detection. The latter possibility is the reason that experiments involving alleles in association, as opposed to dispersion, are more effective (Falconer & Mackay, 1996).

Another noteworthy characteristic of QTLs is that, in addition to additive QTL effects, some QTLs might combine synergistically. Such combination is termed epistasis. Interaction between QTLs has been observed in *Drosophila* bristle number (Long et al., 1995), and more recently in our laboratory in mouse alcohol preference (Tarantino, 1998).

Additionally, many QTLs have been found to affect more than one trait. This result is not a surprise, considering the widespread phenomenon of pleiotropy. For instance, several experiments with Drosophila have found that the QTLs with the greatest

effect for a particular bristle trait also exert their influence on other bristle traits (Long et al., 1995). In regards to alcoholism, the current report presents a case in which QTLs nominated for chronic withdrawal have been shown to affect acute withdrawal.

Finally, QTLs have also been shown to be age-dependent. One example in which a QTL effect was present in mice of a particular age and then disappeared in mice of a different age is reported in McClearn et al. (1998), in which a QTL for alcohol acceptance detected on Chromosome 15 in 100 day old mice failed to replicated in 300 day old mice.

QTL Statistics

Statistically speaking, in detecting and mapping QTLs, a simple t-test for a difference between the means within the classes serves only as an approximation. A t-test is based upon the assumption of a normal distribution of phenotypes within marker class genotypes. In reality, however, the distributions are mixtures of three normal distributions (Falconer & Mackay, 1996).

Instead of a t-test, the data are typically subjected to a maximum likelihood test as follows. A likelihood function is specified in terms of the observed data and the parameters to be estimated. An iterative computer program tests various values for the unknown parameters, and then generates a likelihood function, L, for each trial value. Those values that maximize L are the maximum likelihood estimates of the parameters. The test of significance is the logarithm (base 10) of the ratio L/L_0 (which has a χ^2 distribution), where L is the observed maximum likelihood, and L_0 is the likelihood

computed for the null hypothesis that there is not a QTL segregating (Falconer & Mackay, 1996).

Once a QTL has been determined using the above method, it is usually presented in graphical form as the LOD (for 'log odds') score as a function of chromosome location in cM. Following the convention of human linkage mapping (Lander & Botstein, 1989), those regions of the chromosome displaying a LOD score above 3 (approximately $\alpha = 0.0001$) (Lander & Kruglyak, 1995) are considered significant quantitative trait loci.

Initial QTL mapping experiments localize a QTL to within approximately 20 cM regions. That region shrinks to about 3 cM through progeny testing, and if the region contains markers, fine-scale mapping can narrow down the region with even greater precision (Lander & Botstein, 1989; Paterson et al., 1990). This precision is sufficient to use the QTLs in selective breeding programs, but it is does not identify the actual loci responsible for variation, as is necessary in risk assessment for polygenic human diseases, such as alcoholism.

Where to go with a QTL

Following the methods heretofore described, a QTL is "nominated", or its existence supported by putative scientific evidence. Lander and Schork (1994) propose specific terminology for each step of nomination and replication, which will be discussed later. There are two approaches for identifying the effective gene within a QTL region. The first is positional cloning, and the second is association of variation in the

quantitative trait phenotype using polymorphic markers at candidate loci in the QTL region (candidate gene testing).

Positional cloning is conceptually concise, but procedurally painstaking. In many cases it relies on having a QTL mapped to within 0.3 cM, the approximate size of genomic inserts that can be contained in current cloning vectors (McKusick, 1978). Although the method has been used to identify single loci affecting human diseases, such as Huntington's disease, it has not yet been used to narrow a QTL down to a gene.

The candidate gene approach is the more commonly used method by which to arrive at a gene from a QTL. Candidate genes within the region that significantly associate with phenotypic polymorphisms of the trait in question are understood to affect the trait. However, a QTL need not be discovered in order to test candidate genes that are functionally related to the trait. For instance, researchers have tested candidate genes for a number of human diseases such as heart disease (Sing et al., 1988) and Alzheimer's disease (Corder et al., 1993) without any prior testing of QTLs.

The nomination of candidate genes for quantitative traits like alcoholism relies on the polymorphisms created by functionally significant genes.

Kari J. Buck (1995) notes

> Precise mapping of the chromosome positions of these QTLs should increase our understanding of the genetic causes for individual differences in behavioral sensitivity to alcohol by (1) identifying genomic markers associated with sensitivity to alcohol, (2) allowing the genes specifying behavior to be cloned by position, and (3) elucidating "candidate" genes demonstrating linkage to markers associated with behavioral responses to alcohol.

In conclusion, Falconer & Mackay (1996) note that, "the future for understanding quantitative traits in terms of complex genetics rather than statistical descriptions is bright." Furthermore, the combination of QTL techniques with rodent models will almost certainly allow for the detection and mapping of genes involved with a predisposition to modeled aspects of alcoholism. The approach has met success with other complex traits such as hypertension, diabetes, and epilepsy (Jacob et al., 1991; Rise et al., 1991; Todd et al., 1991).

The Use of Animal Models for Alcohol-Related Phenotypes

Animal models help mitigate the problem of genetic heterogeneity. Additionally, animal models allow for greater experimental manipulation and environmental control than would be ethical in human studies. QTL mapping is much more powerful in animal crosses than in human families due to the sample size involved, and the reduction of non-genetic noise. These advantages make animal models an extremely powerful tool for understanding complex diseases.

For example, the mice used in this study were derived from the DBA/2J and the C57BL/6J inbred strains of mice. DBA/2J mice express severe alcohol dependence and withdrawal, whereas the C57BL/6J mice are characterized by mild withdrawal reactions (Crabbe et al., 1983; Belknap et al., 1993). This conclusion stemmed from Goldstein & Kakihana's (1974) original observation that three inbred mouse strains (BALB/cJ, C57BL/6J, and DBA/2J) differed in ethanol withdrawal severity. Studying such inbred strains and their crosses reduces the problem of genetic heterogeneity (Buck et al., 1997).

Furthermore, the rapid generation time in mice, as compared to humans, allows the collection of greater numbers of observations. Ultimately, this approach provides enough power to detect the influence of individual genes which affect a quantitative trait (Lander & Botstein, 1989).

The second motivation that has turned researchers toward the use of animal models in the study of alcoholism is the need to exercise greater control over environmental factors. Since alcoholism is a complex disease with both genetic and environmental components that interact with one another in a time-dependent nexus, it is very difficult to separate the environmental factors in a randomly breeding population. Animal models provide an alternative by which researchers can control the environment to a greater degree.

A further advantage of the mouse model of dependence is that mice can become physiologically dependent on alcohol in a matter of days, whereas it takes years in humans (Goldstein, 1975). Another advantage is the short generation cycle in mice. The difference between conception and adulthood in mice is only 75 days (Metten & Crabbe, 1996).

One of the greatest advantages of using a mouse model of alcohol dependence is that mapping QTLs in mice leads to the location of analogous human genes without performing any studies on humans (Crabbe, in press). For instance, the steroid 5α-reductase gene found in mice maps to human chromosome 5p15 (Silver & Nadeau, 1997). This bonus comes from the fact that mouse and human genomes are approximately 80% linkage homologous (Copeland et al., 1993).

Finally, understanding the mechanisms underlying alcoholism and alcohol related processes in animal models may eventually lead to more effective therapeutics and diagnostics in humans (Tarantino et al., in press). In fact, "The use of animal models has greatly facilitated the progress of experiments designed to assess the role of genetic constitution in drug sensitivity, tolerance, and dependence/withdrawal (Crabbe, McSwigan, & Belknap, 1985)."

Animal models have proven themselves valuable in the study of human genetic diseases. For instance, the use of animal models helped uncover the genes responsible for single gene diseases such as Waardenburg Syndrome (Delezoide & Vekemans, 1994) and Chediak-Higashi Syndrome (Barbosa et al, 1996). The absence of a complete animal model of alcoholism, however, has made the understanding of this complex disease more difficult.

The ideal and complete pharmacological animal model of alcoholism would encompass each of the following aspects associated with human alcoholism: The animals would voluntarily partake of the drug, by mouth, in sufficient quantities to produce intoxication, tolerance, and physical dependence. The animal would gradually imbibe greater amounts of alcohol, and concurrently learn to stave off withdrawal reactions by increasing alcohol intake. In addition, following an unspecified period of abstinence, the animal would voluntarily return to excessive alcohol use (Goldstein, 1975). This pharmacological model does not even take into consideration the important social aspects of alcoholism such as job failure or arrest record.

A brief discussion of models in general is warranted here. It is important to note that a model is a reduction of the modeled entity in one way or more. For example, a model of an airplane might be quite accurate in terms of the size and shape of the wings in relation to the fuselage, but it is probably much smaller in size than an actual airplane. Furthermore, a complete model is not necessary, because if someone is able to build a complete model of anything, then that person already knows so much about the entity he or she has modeled that it is unnecessary to build a model (McClearn, 1979).

Quite understandably, a complete model has so far proven impossible to achieve, and even a decent approximation would probably be preposterously expensive to maintain. The approach of Deneau, Yanagita, & Seevers (1969) has been the most effective approximation of the ideal alcoholism model. Based on self-administration in rhesus monkeys, Deneau and colleagues modeled a condition in which the animals acquired a physical dependence on the drug, as elicited by withdrawal signs.

Since a complete animal model of alcoholism remains elusive, and the closest approximations tote an outlandish price tag, current methods involve the use of experimental animal models of various components of the disease alcoholism (Goldstein, 1975). Such an approach is valid because alcoholism is understood to be an interaction between various behavioral components (Goldman, 1993).

Without the requirement for voluntary alcohol intake, the model becomes purely pharmacological. In other words, the model reduces the study of alcoholism to specific components, such as alcohol withdrawal, without any regard for how the alcohol is consumed (Goldstein, 1975). Physiological dependence is defined as the manifestation

of withdrawal symptoms after the suspension of alcohol administration (Buck et al., 1997). Withdrawal seizures, in particular, represent a particularly useful measure of alcohol dependence, since they are displayed in all species tested, including humans (Friedman, 1980). Other components include alcohol withdrawal, dependence, acceptance, and preference, among others.

Along with dependence, an array of alcohol-related behavioral responses has already been successfully modeled (Crabbe & Harris, 1991; Crabbe & Buck, 1995; Broadhurst, 1978). These models include initial ethanol sensitivity (McClearn & Kakihana, 1981; Allan, Spuhler, & Harris, 1988; Crabbe et al., 1987(a); Crabbe et al., 1987(b)), sensitization, tolerance, dependence or withdrawal after repeated or chronic exposure (Crabbe et al., 1985; Kosobud & Crabbe, 1986; Crabbe, Feller, & Dorow, 1989), and the perception of ethanol as pleasurable (Erikson & Rusi, 1981; Li et al., 1981; Li et al., 1987). Each of these phenotypes plays a potential role in the underlying susceptibility to alcoholism, and the greater understanding of each could eventually lead to better diagnostics and therapeutics in humans (Buck, 1995). The component under study here is alcohol withdrawal, a representative of physical dependence.

Withdrawal Model

According to the DSM-IV (1994) physiological dependence on alcohol in humans is evidenced by tolerance or symptoms of withdrawal. Specifically, chapter 291.8 in the DSM-IV, titled *Alcohol Withdrawal*, notes that the withdrawal syndrome includes one of the following:

> autonomic hyperactivity (e.g., sweating or pulse rate greater than 100); increased hand tremor; insomnia; nausea or vomiting; transient visual, tactile, or auditory hallucinations or illusions; psychomotor agitation; anxiety; and grand mal seizures.

Withdrawal is a complicated phenomenon, only certain aspects of which can be directly observed. The state of withdrawal could involve altered calcium flux in excitatory amino acid pathways. In addition, EtOH withdrawal is not dependent upon the metabolism of alcohol to acetaldehyde, but is rather a direct result of the alcohol (Crabbe, Merrill, & Belknap, 1991). In addition, a wide variety of dependence-producing drugs share common withdrawal effects on the locus coeruleus (LC) (Gold & Miller, 1992).

Since the degree of physical dependence can not be directly assessed, it becomes necessary to infer it through observations of the outward displays of physical dependence in the form of withdrawal symptoms (Kalant, LeBlanc, & Gibbins, 1971). Likewise, Metten and Crabbe (1996) point out that dependence is operationally defined as the condition displayed upon withdrawal from alcohol.

In using animal models of physical dependence on alcohol, it is important to note the distinction between physical dependence and withdrawal reactions. Physical dependence is a direct effect of alcohol intoxication, physiological in nature and probably located in the brain. Withdrawal symptoms are an outwardly observable surrogate sign of withdrawal, which might differ in different strains even with a similar underlying level of physical dependence (Goldstein & Kakihana, 1974).

Genetic effects on one aspect of withdrawal, such as seizures, may, of course, be distinct from genetic effects on other aspects. The genes influencing one component or another could be different altogether (Horowitz & Dudek, 1983). Nevertheless, various

genetic components of physical dependence exist. Animal studies have demonstrated that nearly every response to alcohol is influenced by genetic factors, and it is unlikely that any aspect of alcoholism's pharmacology will turn out to be free of genetic influences (Crabbe, McSwigan, & Belknap, 1985).

In 1973, Dora Goldstein reported that genes influence alcohol withdrawal severity in mice. Following this statement, she concluded that, "This may mean that vulnerability to physical dependence can be inherited (Goldstein, 1973c)." The heritability of withdrawal syndromes represented the possibility that susceptibility to physical dependence, an integral component of alcoholism in humans, could be passed to offspring genetically. Crabbe, McSwigan, & Belknap (1985) reported that experiments with animal models have demonstrated a significant genetic component on drug dependence and withdrawal.

Several groups have attained success in creating physical dependence on alcohol (as exhibited by withdrawal symptoms) in a variety of animal species, including rhesus monkeys (Deneau, Yanagita, & Seevers, 1969; Winger, Ikomi &Woods, 1970; Woods, Ikomi, & Winger, 1971; Woods and Winger, 1971; Ellis & Pick 1971), cats (Guerrero-Figueroa et al., 1970), rats (Majchrowicz, 1975), and dogs (Essig & Lam, 1971; Ellis & Pick, 1970).

The evolution of the murine withdrawal model

"Experimental crosses of mice and rats offer an ideal setting for genetic dissection of mammalian physiology (Lander & Schork, 1994)." Rodent models appear to be one of

the most valuable approaches in the search for QTLs (Georges, 1997). This section transitions from a discussion of animal models to a specific discussion of the murine withdrawal model.

The first convincing model of physical dependence on alcohol in mice relied on placing both food and water in the same bottle. When the animal is placed on a totally liquid diet, it will consume enough alcohol to remain intoxicated continuously (Freund, 1969). Freund reported the induction of physical dependence on mice, reduced to 65% of their free-feeding weight, after four days of restriction to a sucrose-alcohol diet in which 35% of the total calories were derived from alcohol. Freund's method was the preferred method of induction of alcohol dependence in mice for a few years (Ogata et al., 1971).

Subsequently, Dora Goldstein developed a mouse model for alcohol dependence which rated withdrawal symptoms. The original scale for assessing alcohol withdrawal severity included scoring each animal for signs of lethargy, tremor, tail lift, startle to noise, convulsions on handling, spontaneous convulsions and death (Goldstein & Pal, 1971). This method of scoring required several hours of observation. Subsequently, Goldstein (1972) showed that a correlation coefficient of 0.94 existed between the scores for all seven signs and the single score called "convulsions on handling".

Goldstein published evidence that the convulsion on handling could be reliably scored by different observers, that it was not due to pyrazole injections (utilized to normalize blood alcohol concentrations), and that they did not occur with the same intensity in mice that were not readjusting to a non-alcohol state (Goldstein, 1973a). The measure then evolved further from Dora Goldstein's original array of withdrawal

symptoms to Crabbe's modification of the scale in 1991 (Crabbe, Merrill, & Belknap, 1991). Crabbe increased the measure's resolution by identifying eight seizure levels as opposed to Goldstein's four.

The current model of alcohol dependence as exhibited by withdrawal reactions is based upon Goldstein's measure of the withdrawal reaction, subsequently called "handling induced convulsions" (HICs) (Crabbe, Merrill, & Belknap, 1991). HICs are elicited by holding the mouse up by the tail. If the mouse is undergoing withdrawal, it may arch its back, contort its facial muscles, and jerk or twirl violently (Goldstein, 1971a). The current pharmacological standard for alcohol withdrawal severity in mice is Crabbe's modification. This measure is not, however, unique to alcohol withdrawal. It can be elicited in naive mice with a sufficiently violent spin. Therefore, the reaction might be an exaggeration of a normal reflex (Goldstein, 1972).

In addition, withdrawal after discontinued administration of alcohol and other central nervous system (CNS) depressant drugs has shown handling induced convulsions (HIC, a measure of withdrawal severity) to wax and then wane in parallel with several other behavioral signs of drug withdrawal (Kosobud & Crabbe, 1986; Belknap, Crabbe, & Laursen, 1989; Belknap et al., 1988). Such evidence lends credibility to the use of the HIC withdrawal model in the understanding of a component of human alcoholism.

Goldstein came to the following conclusion regarding her model:

> We believe we have a valid and useful model for alcohol physical dependence. It agrees with other animal models and with what we know about human physical dependence in several respects. The alcohol dose relationships and the time course of the signs that develop after discontinuing the alcohol indicate that we have indeed produced an alcohol withdrawal reaction (Goldstein, 1973c).

Similarly, alcoholism researcher John Crabbe notes, "These withdrawal symptoms define a preexisting state of physical dependence on the drug (Crabbe, in press)."

The mouse model of withdrawal severity is understood to be a measure of physical dependence similar to the seizures experienced during the withdrawal syndrome in human alcoholics (Murray & Berger, 1997). Human alcohol related seizure disorders usually start 12 to 48 hours after drinking ceases, and may occur in persons with otherwise normal clinical EEGs (Maisto, Galizio, & Connors, 1985). In addition, all species studied to date have displayed tremor and convulsions following withdrawal from ethanol (Friedman, 1980; Kalant, 1977).

Acute Withdrawal Model

While early animal models of withdrawal required prolonged (chronic) exposure to the drug, recent advancements have proven short-term (acute) withdrawal-inducing treatments valid as well.

Mice exhibit signs of withdrawal after just a single injection of ethanol, with or without pyrazole treatment. As further evidence that the convulsions are a symptom of alcohol withdrawal, administration of ethanol during the withdrawal period attenuated the animals' response. Some patients control the severity of their withdrawal reaction by reinstituting drinking (Mendelson & Mello, 1985), which is familiarly known as the "hair of the dog that bit you" technique (Maisto, Galizio, & Connors, 1985). The convulsions reappeared another hour or two following re-administration (Goldstein, 1971b). Furthermore, mice have shown withdrawal signs while still intoxicated. Similarly,

alcohol withdrawal states in humans occur when blood ethanol levels have not returned to the normal physiological condition (Mendelson & Mello, 1985). This short-term withdrawal is an index of physical dependence (Goldstein, 1972).

Physical dependence on alcohol decays extremely rapidly, especially in mice. The cycle of intoxication used in many studies is analogous to a single bout of drinking in humans. Furthermore, the withdrawal reaction in mice has been shown to reflect the intensity of the previous binge of drinking, and not on the entire history of drinking (Goldstein, 1975; Goldstein, 1973c). Such is also the case in humans (Victor, 1970).

The above observations, coupled with McQuarrie and Fingl's (1958) observation that a single oral dose of EtOH elevated seizure thresholds in albino mice, paved the way for Kosobud and Crabbe's (1986) experimentation, which showed that withdrawal seizure prone (WSP, mice selectively bred to exhibit severe withdrawal reactions) and withdrawal seizure resistant (WSR, mice selectively bred to exhibit mild withdrawal reactions) mice differ in their withdrawal reaction to a *single acute dose* of ethanol (4.0 g/kg, intraperitoneal) in a similar manner to their difference in chronic alcohol withdrawal. These results demonstrate that a mild state of physical dependence and withdrawal can be elicited by a single exposure to ethanol (Crabbe, Merrill, & Belknap, 1991). Therefore researchers created a model of alcohol withdrawal that did not require pyrazole injections, and could be carried out on a single day (Belknap et al., 1993).

The validity of this model relies, in part, on previous studies that have shown that genetic differences in acute withdrawal liability in humans might be related to a risk for the development of alcoholism (McCaul et al., 1991). Furthermore, the fact that sons of

alcoholics report greater hangover symptoms (which are a representation of an acute withdrawal syndrome) than sons of nonalcoholics lends credence to the acute withdrawal model of an alcoholism component (Newlin & Pretorius, 1990).

There are several advantages to the acute withdrawal model over the previously standard chronic model. First, the dependence on alcohol can be induced rapidly. Second, the process by which dependence is induced is not as injurious as chronic induction, thus the animals may be repeatedly studied. Finally, the animals can be studied for various other phenotypes allegedly related to alcohol withdrawal, such as altered levels of drug self-administration, after acute withdrawal testing (Crabbe, Merrill, & Belknap, 1991).

The RI Nominees for Withdrawal QTLs

The initial nomination of QTLs often involves RI strains, mainly because genotyping is unnecessary (Tarantino et al., in press). In addition, although RI strains were developed to detect major gene effects (Bailey, 1971; 1981), they act as an excellent resource for identifying QTLs for complex traits (Gora-Maslak et al., 1991). Furthermore, RIs possess all the advantages of inbred strains (such as the accumulation of marker data) (Bailey, 1981).

The C57BL/6 (B) and DBA/2 (D) lines of mice form an excellent study cross (BXD) based on the fact that C57's, unlike DBA mice, show almost no convulsions on handling (Goldstein, 1975). Taylor's (1989) BXD set is currently the most commonly used RI set because it has the largest marker database (about 1500) and the greatest

number of strains (26) (Belknap et al., 1996). A number of researchers have nominated alcohol withdrawal QTLs using RI strains.

The heritability for alcohol withdrawal was estimated in the RI data (additive effects) to be 0.19 (Belknap et al., 1996), which was in close agreement with the heritability estimate of 0.26 from an earlier experiment for chronic withdrawal severity (Crabbe et al., 1985). A more recent estimation by Crabbe (in press) raises the narrow-sense heritability to 0.55.

Buck et al. (1997) published results from studies in which they nominated QTLs for alcohol withdrawal using 26 BXD RI strains of mice. The process included testing 288 adult male mice for alcohol withdrawal, using Crabbe's previously described HIC method. Of the 26 strains available, 21 had sample sizes sufficient to perform tests. For each of the markers available in the MAP MANAGER BXD marker database, arbitrary values were assigned to each strain according to the following algorithm: strains which contained two copies of the C57BL/6 (B6) were scored 0, and those bearing two copies of the DBA/2 (D2) allele were scored 1.

Subsequently, correlation coefficients (r) were determined between the strain means for residual alcohol withdrawal severity and the markers. The p values reported for each marker were determined using linear least squares statistical procedures rather than LOD scores, which are applicable for F_2 data. There is little loss of statistical power when the markers are within 10 cM of the QTL (Darvasi et al., 1993).

In these initial RI QTL studies for acute withdrawal, markers associated with increased risk for withdrawal clustered within discrete chromosomal regions on

chromosomes 1, 2, 4, 6, 8, and 11 ($p < 0.05$) (Buck et al., 1997). A QTL at approximately 75 cM was highlighted on Chromosome 1. Two putative QTLs on Chromosome 2 associated with markers at about 28-38 cM from the centromere and 75-85 cM. Chromosome 6 contains a QTL at approximately 40 cM. The QTL on Chromosome 11 is in close proximity to $GABA_A$ receptor genes. Finally, a QTL on Chromosome 4 at about 40 cM from the centromere associated with increased to alcohol withdrawal severity. This particular QTL, linked to the *Tyrp1* locus (Buck et al., 1997), is of particular importance as it is the QTL under confirmatory study in this research.

Similar studies for chronic withdrawal nominated 16 chromosomal regions as QTLs ($p < 0.05$). Ten of those regions, including those on chromosomes 1, 3, 4, 9, 11, 13, 15, 17, and 18, the D2 allele was characterized as the "increasing" allele, so that animals homozygous for the D2 allele at that locus displayed higher withdrawal severity than heterozygotes or B6 homozygotes. At the remaining 6 putative QTLs, including those on Chromosomes 2, 11, 12, 13, 14, and the X Chromosome, the B6 allele was the increasing allele (Tarantino, McClearn, & Plomin, 1997). The QTL nominated by Tarantino, McClearn, & Plomin (1997) was also associated with the *Tyrp1* locus. This nomination represented the second nomination of a QTL at this location, each by an independent laboratory.

Crabbe subsequently reported another nominative study for chronic withdrawal QTLs, which highlighted 10 chromosomal regions at $p < 0.01$. These regions included parts of Chromosomes 1, 3, 9, 10, 12, 13, 15, and 18. Multiple regression analysis

determined that the four most influential of these ten putative QTLs, taken in aggregate, controlled 86% of the genetic variance for chronic withdrawal severity.

Interestingly, analysis of control groups in this study revealed a QTL at Chromosome 4, among others (Crabbe, in press). Furthermore, only one of the QTLs nominated by Buck in 1997 showed up in Crabbe's analysis. This reflects the fact that not all of the genes determining acute withdrawal are shared as determinants of chronic withdrawal.

A study by Belknap et al. (1993) reported six regions as potential QTLs for acute withdrawal at the $p < .05$ level. The regions included parts of Chromosomes 1, 2, 4, and 7. The strongest association presented was that for the *Ly-20* region of Chromosome 4. As should be expected, this region represents the same area of Chromosome 4 that had previously been nominated in independent studies, at about 55 cM from the centromere.

On the other hand, Gora-Maslak (1991) reported no QTL on Chromosome 4, though this study was for chronic withdrawal. The regions nominated in her study ($p < 0.05$) included portions of Chromosomes 1, 3, 12, and 13.

Methods of Replication

An important concern in QTL studies is the need to distinguish true positive results from false positive results (McClearn et al., 1997). The p values calculated for a single comparison do not provide adequate protection against type I errors when making multiple significance comparisons (Miller, 1981; Rice, 1989). For example, Monte Carlo simulations of QTL mapping studies that use RI strains and a critical p of 0.01 indicates

that 1.4 ± 1.1 false positive QTLs are detected in a typical QTL study (Belknap & Mitchell, 1995).

Crabbe concluded that about half of the 11 associations he reported (in press) would turn out to be false positives. Tarantino, et al. (1997), note that false positives are one of the main concerns of the RI QTL method. This concern is due to the large number of statistical tests employed. There are two methods by which to limit type I errors (false positives). The first is to employ a stringent level of statistical significance for the nomination of QTLs (Lander & Kruglyak, 1995). The second is to follow up nomination studies with replication, in which case a relaxed alpha ($p < 0.05$) is allowable (Tarantino et al., 1997).

This two step approach takes into consideration the consequences of both type I (identification of a false QTL) and type II (failure to identify a QTL) errors (Belknap et al., 1996). Buck, et al. (1997) are among the many researchers who use confirmatory studies rather than stringent p values. The reason is that the latter could "lead to inflated type II errors and potential failure to identify important QTL,...(Buck et al., 1997)." Belknap et al. (1996) suggest independent experimental confirmation rather than requiring p values as stringent as $p < 0.00002$.

Another good argument for the replication of QTL nomination experiments is that some QTLs might be missed if the two strains (or populations) under study happen to be fixed with alleles for similar effects. Using the two step approach to QTL confirmation, researchers can navigate the canyon between type I error protection and the need for statistical power (Belknap et al., 1996).

Lander & Kruglyak (1995) propose a framework for reporting linkage in the literature as follows. *Suggestive QTL* – statistical evidence expected to occur once at random in a genome scan. *Significant QTL* – statistical evidence expected 0.05 times in a genome scan. *Highly significant QTL* – evidence expected to occur 0.001 times in a genome scan. *Confirmed linkage* – a significant or highly significant QTL that has been replicated in an independent study at a nominal *p* of 0.01. This study has taken a significant QTL and attempted to confirm it through genotypic selection, the last of the four confirmatory methods to be presented here.

F_2 Intercross Studies

The primary method utilized to date to replicate a nominative QTL study is through F_2 intercross studies. Most researchers consider the F_2 study the second stage of QTL studies (Metten & Crabbe, 1996). Many researchers consider an F_2 study a prerequisite for follow-up confirmatory research. For instance, in 1993 Belknap presented his intentions of testing the BXD chromosome mapping results in a segregating F_2 population from a C57BL/6 X DBA/2 cross. "Until this is done," he illuminated, "the present BXD results should be regarded as provisional (Belknap et al., 1993)."

Furthermore, the results of QTL nomination by Buck et al. (1997) were not reported except in conjunction with the F_2 data. The replication involved testing 451 B6D2 F_2 intercross mice of approximately equal numbers of males and females for acute withdrawal. The mice were then sacrificed, and the DNA was extracted from their livers using a salting-out method adapted from Miller, Dykes, & Polesky (1988). The mice

were genotyped based on a method by Dietrich et al. (1992) and Serikawa et al. (1992). The mice were genotyped at the *Tyrp1* locus (Silvers, 1979). The mice were given a score of 0, 1, or 2 for each marker tested, based on the gene dosage of the D2 alleles. Correlation coefficients (Pearson's *r*) were determined. One tailed *p* values were reported based on the directional implications from the RI study.

The results of the F_2 intercross study supported the nomination of the putative QTLs on Chromosomes 1, 2, 4, and 11, while rejecting those on Chromosomes 6 and 8 (Buck et al., 1997).

Likewise, Tarantino, McClearn, & Plomin (1997) reported F_2 confirmatory studies in conjunction with BXD RI results. Those chronic withdrawal QTLs identified in the RI study that were confirmed in the F_2 intercross study include those on Chromosomes 1, 4, 15, 17, and X. In addition, a QTL on Chromosome 2 was nominated in the RI study and the F_2 intercross study, but the increasing alleles were different in each case.

Tarantino's F_2 intercross study provided a replication rate of 25% (4 of 16), which is consistent with the expected replication of at least 25% of the QTLs nominated in the RI (Belknap et al., 1996). The replication rate also falls in line with the 28.6% reported in a similar study of alcohol preference, in which F_2 intercross studies provided replication (Tarantino, in press).

In regard to the Chromosome 4 QTL, Tarantino reported that it had a peak LOD score of 3.7 at approximately 52 cM. The one LOD support interval spanned from 32 to 59 cM. This QTL fits best in the additive or dominant models. Importantly, the support

interval included both the QTL identified in her RI analysis as well as the acute QTL linked to the *Tyrp1* locus identified by Buck et al. (1997).

With power set at 0.9, about 300 B6D2 F_2 mice must be tested to detect QTLs with effects accounting for more than 25% of the genetic variance for traits with a heritability (h^2) of 0.3 (Buck, 1995). Selective breeding estimates suggest that about 30% ($h^2 = 0.3$) of the phenotypic variance of acute withdrawal is genetically determined (Crabbe et al., 1985). The heritability is potentially higher, as Belknap et al. (1993) reports a value of 0.34.

Knockout Preparations

The allure of "gene knockout" mice as a replication tool is that, by creating a mouse devoid of a particular gene, it is possible to visualize the action of that gene when it is present. Saudou et al. (1994), for example, have created a knockout mouse devoid of the serotonin 5HT1B receptors. This method provides a means by which to test the action of the receptors directly (Buck, 1995). Researchers should be able to proceed with making knockout preparations for other candidate genes.

However, the effect of the gene could appear different against various background genotypes. The QTL could even disappear if bred into a different genetic background. Perhaps other genes with a similar role could compensate for the lost effect of the knockout gene. This possibility highlights the important role of various interacting genes in any complex trait, particularly alcoholism (Lander & Schork, 1994).

In addition to the knockout method, Buck (1995) has proposed the use of a "knockdown" method. By making use of antisense oligodeoxynucleotide strategies on the candidate genes nominated in this (and other) studies, researchers will be able to observe the quantitative effect of certain genes on behavioral phenotypes (Wahlestedt, 1994). For example, Silver, Nadeau, & Goodfellow (1996) report the successful use of the knockdown procedure in NMDAR1, whereas the knockout of this gene proved lethal.

Short Term Phenotypic Selection

Not only did Buck et al. (1997) withhold reporting their nominated QTLs until after F_2 testing, they also reported the results of a short term phenotypic selection concurrently. In that study, mice from selectively bred lines for high alcohol withdrawal severity (HAW) and low alcohol withdrawal severity (LAW) of approximately equal numbers of males and females were tested for withdrawal and genotyped. The D2 allelic frequency (q) was determined for each of the lines, and the difference between the two (q_{LAW}-q_{HAW}) was also determined. A statistical test based on z (normal variate) was computed (Belknap et al., 1997). This replicative study provided further confirmation for the QTLs on Chromosomes 1, 4, and 11, while rejecting those on Chromosomes 2, 6, and 8 (Buck et al., 1997). None of these QTLs evinced gender differences.

Buck's (1997) short term phenotypic selection study once again highlighted a QTL for alcohol withdrawal severity on Chromosome 4. This finding represents the third nomination of the QTL. Moreover, F_2 mice homozygous for the D2 allele at D4Mit186 showed increased susceptibility to acute alcohol withdrawal ($p = 0.02$).

Crabbe (in press) has announced that he intends to use short term phenotypic selection to confirm the 10 withdrawal QTLs nominated in his most recent study. By examining the genotypes of the withdrawal seizure prone (WSP) and withdrawal seizure resistant (WSR) lines of mice, it is possible to derive support for the nominated QTLs. If the WSP mice have significantly different allelic frequencies than the WSR mice at the location of the QTL in question, then this supports the linkage for that QTL.

Genotypic Selection

The above methods of replication require the examination of independent segregating populations or the construction of congenic lines differing only in the QTL of interest. Such methods demand a minimum of several years and thousands of marker assessments (Bennett, 1997). A quicker, more cost-effective method has been proposed. This method, hereby referred to as genotypic selection, could even replace some of the other methods of replication.

Although the application of another method in order to re-confirm a QTL might seem like overkill, the methods and multiple models used in initial nomination beckon more confirmatory studies. For example, a LOD score of 3.0 would no longer be acceptable proof of linkage, and even a LOD score of 4 or higher might not be sufficient (Devor, 1994). Belknap and colleagues (1996) note that a p value of 0.0001 (LOD 3.3) is necessary to provide 95% protection against even one false positive QTL in BXD RI studies. Lander & Schork (1994) go further, recommending 0.00002 (LOD 3.9), although they make several "worst case" assumptions regarding the QTL nomination.

Fortunately, genotypic selection presents a simple, cost-effective, efficient method by which to confirm individual QTLs. By genotyping live animals from a DNA sample derived from a tail snip, animals can be selectively bred for a single risk-altering QTL, and their progeny subsequently tested phenotypically to determine the effect of the QTL in question (Metten & Crabbe, 1996). This process is variably termed "genotypic selection" (McClearn et al., 1997), "marker-based selection" (Dudek & Tritto, 1995), or "segregating congenics" (Bennett et al., 1997).

Bennett described the classical process of congenics (1997), in which individuals of the desired phenotype (donor strain) are repeatedly backcrossed to an inbred mouse from a different strain (recipient strain). After 10 generations of backcrossing, less than 0.2% of the donor genome that is unlinked to the genes responsible for the phenotype remains (Silver, 1995). This process of congenics has been virtually outmoded due to the advent of genome-wide microsatellite markers in mice, and thus the possibility of genotypic selection (Bennett et al., 1997).

In genotypic selection mice are made homozygous for a single QTL in one generation (Bennett et al., 1997). First, the mice are genotyped for markers flanking the QTL to be confirmed. Those animals that are homozygous for all markers in the region are chosen as parents for the next generation of mice. The mice homozygous for the risk-increasing alleles (C57BL/6) at the QTL are mated together, so that their offspring are homozygous for the QTL, while alleles at all other loci remain polymorphic. Concurrently, those homozygous for the contrasting allele (DBA/2J) at the locus are bred together. The two contrasting offspring lines are then phenotyped, and a significant

difference between the two provides further evidence for a true positive QTL (Crabbe, in press). The method has been used to confirm QTLs for loss of righting reflex (Bennett et al., 1997), and for alcohol consumption (McClearn et al., 1997) in mice.

Some of the obvious advantages of genotypic selection as a confirmatory method include: 1) the parents of the generation to be tested can be identified with relatively little genotyping, 2) these parents need not be phenotyped, and 3) the offspring to be tested phenotypically need not be genotyped (McClearn et al., 1997). In genotypic selection, a number of potential parents are genotyped at a particular location, using 2 or 3 markers (depending on the size of the region under examination). All of the animals are genotyped at one of the markers, and those animals that are heterozygous can be removed from the pool of potential parents. Those animals that are homozygous are genotyped at the next marker. Once again, all homozygous animals are weeded out, as are those animals that are homozygous for a different allele than that for which they were homozygous at the other marker. Those remaining potential parents are genotyped at the third marker, and once again separated.

Those animals that are homozygous for either the allele which should increase or decrease the trait in question at all three markers are potential parents from which to choose mating pairs. Increasing homozygotes are mated with increasing homozygotes, and vice-versa. Only the progeny of these matings need be tested phenotypically. If the region in question is a true QTL, then the progeny of the "increasing" matings should have a significantly higher phenotypic score than the progeny of the "decreasing"

matings. It is not necessary to phenotype the progeny of the genotypically-selected matings.

One of the few drawbacks to genotypic selection is that the phenotype to be tested will become more variable due to the increased genetic variance brought about by segregating loci (Bennett et al., 1997).

Overall, however, genotypic selection has already proven to be a valuable tool in QTL research. Furthermore, it has been recommended as the method of choice for QTL confirmation (Bennett et al., 1997). Chapter 3 presents a confirmation of the QTL on Chromosome 4 using genotypic selection.

Chapter 3

GENOTYPIC SELECTION FOR ACUTE ALCOHOL WITHDRAWAL SEVERITY IN MICE

The objective of the study was to confirm or refute the existence of the putative QTL on Chromosome 4, with peak LOD location at 53 cM and a 1-LOD support interval ranging from 35 to 62 cM (Tarantino, McClearn, & Plomin, 1997).

Methods and Materials

Genotyping

Following standard protocol, the genomic DNA was extracted from the tail tips of 303 F_3 mice derived from a cross between C57BL/6 and DBA/2 inbred lines of mice. The extraction involved clipping a small (≈10mm) portion of the mouse's tail, and then digesting the tail clipping in a 600 µl buffer/17.5 µl proteinase K solution for at least 8 hours at 55° Celsius. Following digestion, each individually labeled digested tail received 300 µl phenol and 300 µl chloroform to separate the residue from the DNA.

The resulting mixture was thoroughly vortexed and then centrifuged for 5 minutes to separate the DNA solution from the wash. The top layer (DNA) was placed in new vials, into which was added 2 volumes of EtOH, which precipitated the DNA out of

solution. The precipitated DNA was dried of the EtOH, and dissolved in 100 µl of TE buffer (pH = 8.0) and refrigerated for storage.

The DNA was then genotyped at each of three markers. The markers were chosen, based on information from the MIT database (http://www-genome.wi.mit.edu/cgi-bin/mouse/index/) and the Mouse Genome Database (MGD, 1998), as those markers flanking the region hereby under experimental confirmation (Chromosome 4, 37.5-60 cM). In addition, we chose a third marker near the middle of this region (48.5 cM) to detect recombination between the two flanking markers. Figure 3-1 portrays the markers used. The primers for these markers were purchased from Research Genetics.

The genotyping protocol required a PCR reaction involving the above primers. The reaction mixture was 12.5 µl total volume consisting of 30 ng genomic DNA, .4 µM primers, .2 mM of each dNTP, 0.5 U Taq polymerase (Perkin-Elmer Cetus), 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and .16 mg/mL Bovine Serum Albumin. A Perkin-Elmer 9600 thermocycler carried out the DNA amplification. The thermal cycling process included a 5-minute denaturation step at 94° C, 35 cycles of 30 seconds each at 94° C, 30 seconds at 53° C, 30 seconds at 72° C, and a final extension step for 10 minutes at 72° C. The products were separated on 4% agarose gels and subsequently stained with ethidium bromide.

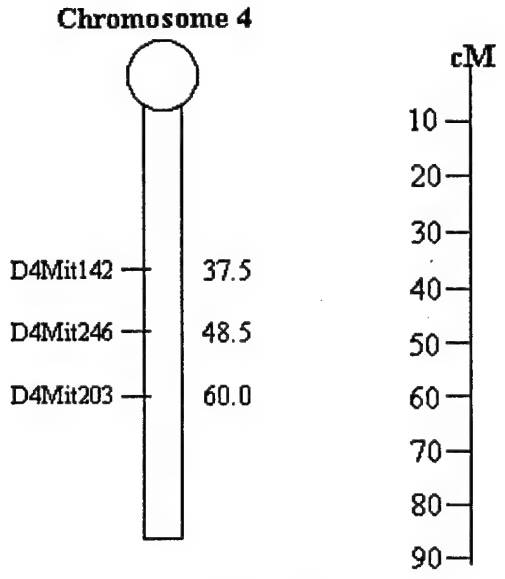


Figure 3-1

The markers genotyped for the selection. The middle marker was chosen due to the possibility of recombination between the two flanking markers.

Animal Mating and Husbandry

Animals homozygous at each of the three markers for either alleles which increase the severity of withdrawal (increasing) or those which decrease the severity of withdrawal (decreasing) were put into a pool of potential parents for the genotypically selected generation. Ten pairs of regionally homozygous "increasing" parents were mated, as well as ten pairs of regionally homozygous "decreasing" mice.

The offspring of these matings, who were almost guaranteed homozygous (barring mutation) increasing or decreasing at 37.5-60 cM on Chromosome 4, were weaned at about 23 days of age, housed individually, and fed a diet of Purina Mouse

Chow 5010, of which they partook *ad libitum*. The environment was maintained at 72 ± 2 °C, with a light and dark cycle of 12 hours. All procedures were approved by the Pennsylvania State University Animal Care and Use Committee (approval #98R-013-0) in accordance with USDA and USPHS guidelines.

Amy Lake, an AALAS accredited animal technician, was responsible for the mating, weaning, and general animal husbandry prior to testing.

Phenotyping

Mice were phenotyped at about 8 to 12 weeks of age, which required 8 separate batches. A batch is a group of about 20 animals, all of which who were old enough for testing during the same general time. For example, the first genotypically selected mice were born on December 19th, 1997, almost four months before the last batch (March 12th, 1998), so that when the first batch reached testing age, the 8th batch had not even been born yet, and even the second batch was about 2 weeks younger than the standard testing age.

Phenotyping involved injecting the animals with a high dose of ethanol (4 gm/kg, 20% v/v in saline, intraperitoneal), just after lifting the animal by the tail and assessing the baseline handling induced convulsion according to the scale published by Crabbe, Merrill, & Belknap (1991). These convulsions range from none (score = 0) to spontaneous and severe, possibly resulting in death (score = 7).

Two hours after this injection, the mice were again lifted gently by the tail and scored for handling induced convulsions. This testing proceeded every hour through 12

hours after the initial injection. The supra-baseline scores from hours 2 through 12 were then summed to give the index of withdrawal severity called withdrawal area under the curve (WAUC).

Statistical Analysis

The index of withdrawal severity (WAUC) was not normally distributed. In order to solve this problem, we began by attempting a number of transformations, including log transformations and a square-root transformations. None of these transformations succeeded in creating a normally distributed index of WAUC. In order to obtain a normally distributed index, we created a variable that removed "batch effect" as follows.

A significant difference was detected between mean WAUC scores between the batches. This difference is considered a "batch effect". In order to remove this potentially confounding factor, a series of indicator variables were created based on which batch each mouse was tested in. A mouse from batch eight was tabulated as a 0 for each of the seven indicator variables, while a mouse from batch x (besides 8) was scored as 1 for indicator variable x, and 0 for the rest. A regression of WAUC on batch indicator variables and genotype stored residuals that were normally distributed. The resulting residuals removed all of the effect on the withdrawal scores that testing in batches had. In order to replace any batch effect confounded with the genotype effect (or the differences in WAUC that we were looking for), the predicted value from a regression of WAUC on genotype was added to the residual.

After removing the effect of testing in batches, a t-test comparing the mean WAUC (batch effects removed) for each of the two genotypes (C57 scored as 0, DBA scored as 2) was performed using SPSS software for Windows. The result of this test is the most important finding of our research, and is discussed in greater detail in the following section.

Results

A one-tailed t-test for a difference between the means of the two genotypes revealed that the QTL in question significantly influenced acute alcohol withdrawal in mice ($p = 0.0005$ $n = 161$). One-tailed logic was utilized due to the highly directional nature of the test. As seen in Figure 3-2, the mean withdrawal scores were higher for those mice homozygous for the increasing allele than for those mice homozygous for the decreasing allele. The mean WAUC for the mice bred for the increasing QTL was 3.51 (Standard Error of the Mean = .52, $n = 89$), while the mean WAUC for the mice bred for the decreasing QTL was 1.54 (S.E.M. = .29, $n = 72$).

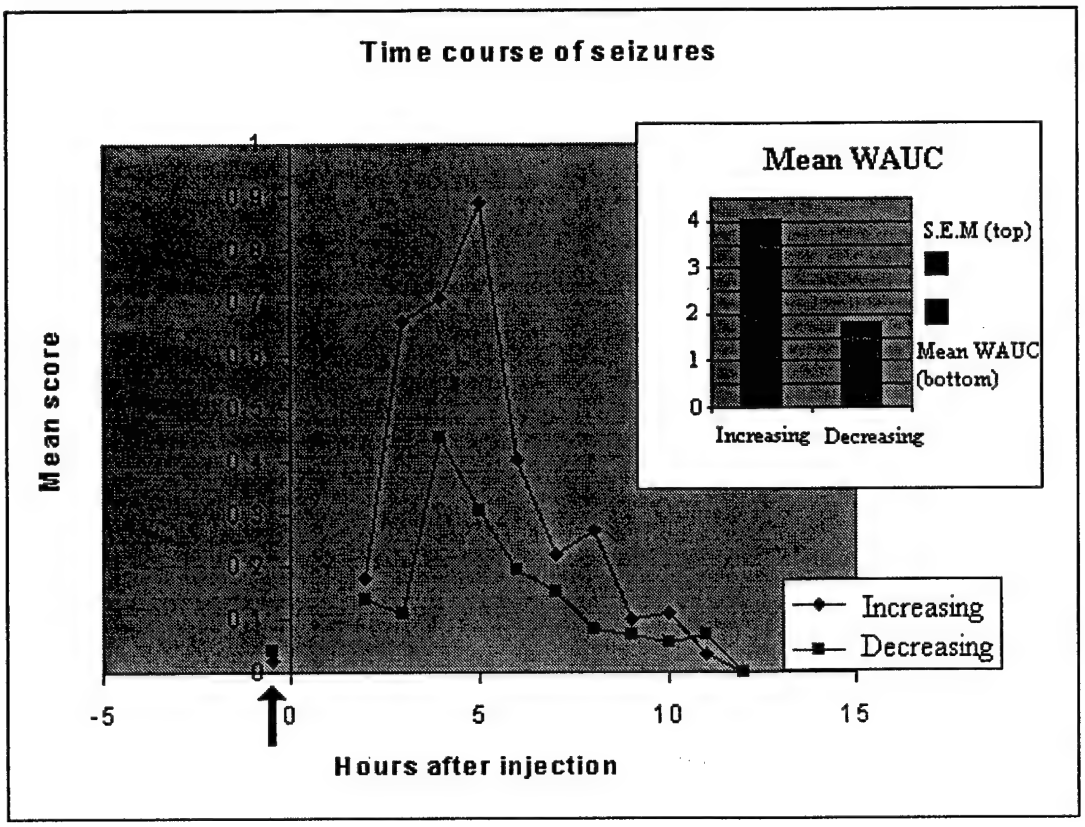
Figure 3-2 also shows the time course of the withdrawal seizures. The withdrawal seizures peaked at 5 hours after injection for the mice with the increasing QTL. The mice with the decreasing QTL exhibited a peak withdrawal reaction at 4 hours following injection, on average.

In addition, to ensure that the difference in mean WAUC scores between each genotypic class did not stem from the sizes of families produced, I also analyzed the difference between mean family WAUC. In other words, there was a possibility that

there was a difference between the two genotype's mean WAUC scores only because perhaps a family of mice that produced the most severe withdrawal reactions just happened to be of the type 2 genotype. In order to remove the effect of family size, I performed a t-test of the difference between the two genotypes taking into account only 20 WAUC scores (the mean WAUC of each of the 20 families). The results were still significant at the level of $p = 0.01$. The family mean WAUC for the increasing genotype was 3.8 (S.E.M = .9341) and that of the decreasing genotype was 1.2 (S.E.M. = .2711).

Figure 3-2 Seizure differences

The time course of the average seizure at each hourly measurement is shown. The arrow indicates the injection of ethanol. The baseline HIC average is shown before hour 0. Furthermore, although measurements were not made, the average HIC usually drops immediately after administration of ethanol (Goldstein, 1972). The inset graph shows the mean area under the curve (WAUC) for those animals with increasing alleles vs. decreasing. The standard error of the mean is stacked on top of the mean bar.



Discussion

The results of this study indicate that the QTL for acute alcohol withdrawal on Chromosome 4 is a true positive QTL for acute alcohol withdrawal severity. The group of mice homozygous for the increasing allele showed more severe alcohol withdrawal reactions than the mice homozygous for the decreasing allele, when the rest of the genotype was left to segregate independently.

Furthermore, this confirmation of a QTL for acute alcohol withdrawal adds credence to the use of genotypic selection as a means by which to confirm QTLs in the multi-step process. This process minimizes both type I and type II errors.

It is important to note, however, as Goldstein warned in 1975, that we have not found a QTL for "addictability", but only one that affects withdrawal seizure severity. Buck et al. (1997) agree, stating that "the phenotype examined in the present studies represents an animal model of acute physiological dependence liability, rather than a model of alcohol dependence." Until the discovery of the underlying biochemical change that occurs in physically dependent animals, actual physical dependence can not be measured. It is even possible that the biochemical physical dependence is identical in C57BL and DBA mice, but that DBA mice express it more easily in the form of convulsions (Goldstein & Kakihana, 1975).

Other Withdrawal-Influencing Drugs

Several sedative drugs initially suppress withdrawal seizures in a manner similar to alcohol (Goldstein, 1975b). Furthermore, a genetic cross-dependence between ethanol and phenobarbital, among other drugs, exists (Crabbe, McSwigan, & Belknap, 1985). Such evidence attests to the applicability of testing components of alcoholism through seemingly isolated studies. Researchers might be learning about dependence on more than just alcohol.

Using HICs as the primary withdrawal sign, researchers have observed physical dependence on nitrous oxide (Smith et al., 1979; Ruprecht et al., 1983; Belknap, Laursen, & Crabbe, 1987). Nitrous oxide and other drugs that produce HIC upon withdrawal all have relatively rapid rates of elimination (Eger, 1985; Smith et al., 1979; Wollman & Dripps, 1970). Interestingly, surgical anesthetics with rapid elimination rates in humans

are known to produce hyperexcitability withdrawal reactions more so than drugs with slower elimination (Smith et al., 1979; Harper et al., 1980; Price & Dripps, 1970). This observation raises the possibility that a predisposition to greater ethanol withdrawal shares genetic determinants with other drugs (Belknap et al., 1993).

Therefore, the elucidation of the genetics of alcohol withdrawal severity promises also to shed light on withdrawal from other drugs. Researchers making headway in even reduced components of alcoholism research may be opening doors throughout health science.

Candidate Genes

As previously noted, gaining an understanding of the genetics of alcohol withdrawal does not mean we have also reached an understanding of its biochemical causality. However, this study highlights some of the valuable directions in which such understanding might be found. Hypotheses regarding the biochemical basis of withdrawal suggest certain candidate genes within the QTL. Some of those hypotheses follow.

This QTL probably has a functional effect on the central nervous system, rather than acting through dispositional mechanisms such as metabolic rate, absorption rate, or distribution (Crabbe, McSwigan, & Belknap, 1985). Crabbe came to this conclusion because the blood ethanol concentrations in various withdrawal tests did not differ within either sex or replication. Nevertheless, in the search for candidate genes, none of the genes involved with alcohol metabolism have yet been ruled out.

Reduced risk for withdrawal convulsions could be associated with reduced extracellular glutamate levels (Buck et al., 1997). In agreement, ethanol treatment results in increased glutamate uptake in rat brain (Foley & Rhoads, 1992). These rudimentary theories, combined with the knowledge of the action of certain genes in this QTLs region, encourage testing candidate genes, to see if they are responsible for the variance that this QTL produces.

The QTL hereby confirmed overlaps loci identified for various other seizure phenotypes, including mouse models for epilepsy. For instance, *Asp2* on Chromosome 4 has been identified as a gene influencing audiogenic seizures (Neumann & Collins, 1991). Other candidate genes within the region include *Grik3* (a glutamate receptor gene), *Ssdh1* (succinic semialdehyde dehydrogenase), and *Bis1,* a β-carboline-induced seizure gene. (Buck et al., 1997). Crabbe (in press) nominated a cluster of genes as candidates, including Shaker related potassium channel genes (*cort*, *Kcnab2*), *Bis1*, and the gene for the zinc transporter 2 (*Znt2*). Tarantino, McClearn, & Plomin (1997) also reported *Grik3* as a candidate gene, and added *Htr1d*, a serotonin receptor. Figure 3-3 displays the location of each candidate gene on Chromosome 4. A discussion of the region labeled "Tarantino selection" will follow.

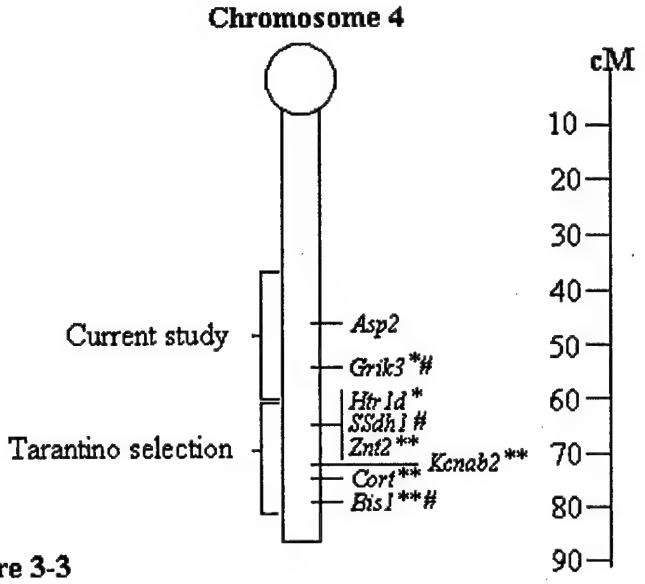


Figure 3-3

Candidate genes within the chromosomal region of the QTL in this study as well as within the region that one member of each mating pair had been selected for previously by Tarantino. *Nominated by Tarantino et al., 1998 **Nominated by Crabbe, In press. #Nominated by Buck et al., 1997. *Asp2* is my nominee.

The audiogenic seizure prone gene *Asp2*, located at 45.1 cM from the centromere, is part of the 3-member family of genes affecting the inheritance of seizure susceptibility. Intense auditory stimuli have been shown to induce a behavioral pattern of wild running followed by a clonic seizure and then a fatal tonic seizure (The Mouse Genome Database (MGD), 1998). The DBA/2 inbred strain is the prototypically seizure-susceptible line, while the C57BL/6 is the prototypically seizure-resistant line. Although the withdrawal seizures elicited in this study are thought to be independent of other types of general seizure activity, it is possible that *Asp2* is the gene that influences HIC in ethanol-withdrawn mice. The possible mechanism is that the *Asp2* gene increases the DBA/2J line's "excitability plasticity" more than it does that of the C57BL/6J line. This hypothesis undergoes a more detailed discussion later.

Grik3, an ionotropic kianate glutamate receptor, located 57.2 cM from the centromere, has been implicated in multiple studies as well. Glutamate receptors, which constitute a major component of the excitatory transmitter system, are located throughout the mammalian brain. Brain damage caused during epileptic seizures can be largely attributed to excessive activation of neurons via glutamate (MGD, 1998). This particular kainate glutamate receptor is homologous with the human gene *GRIK3*, which maps to Chromosome 1p (Puranam et al., 1993).

Besides the kainate receptors (*Grik-* genes), exist the N-methyl-D-aspartate (NMDA) receptors, or *Grin-* genes. Although no *Grin-* genes have yet been mapped to Chromosome 4 in the mouse, they are of particular interest because NMDA receptors have been implicated in the biochemical seizure mechanism in WSP and WSR mice (Crabbe, Merrill, & Belknap, 1991).

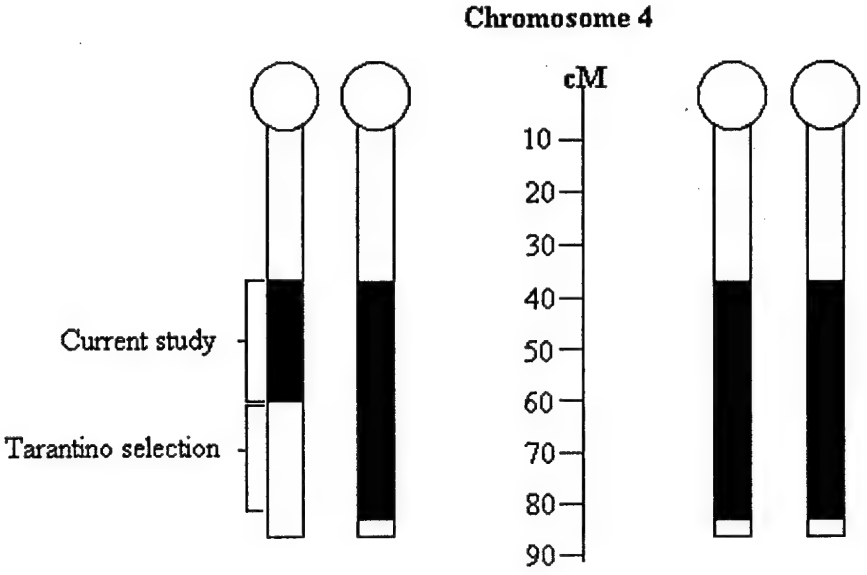
In 1991, McClearn et al. reported their fascination at the correlation of 0.51 between *Adh*-1 (the Chromosome 4 mouse homologue of human ALDH-2), and low dose activating effect of ethanol. Subsequently, *Adh*-1 was withdrawn from the literature. However, the gene was later renamed *Adh1*, withdrawn again nearly a year later, only to be renamed *Ssdh1*, for succinic semialdehyde dehydrogenase (MGD, 1998). Succinic semialdehyde dehydrogenase controls electrophoretic variation in the mitochondrial AHD isoenzyme AHD-1 (MGD, 1998). Arguing against this gene, a study by Goldstein (1975) showed that drugs that affect acetylcholine or serotonin did not alter the ethanol withdrawal reactions, as other drugs did.

Ssdh-1 is located 66.1 cM from the centromere on Chromosome 4, which is outside of the chromosomal range of the current study. However, a significant portion of the chromosome adjacent to the marker region is likely to segregate with the QTL. It might be valuable to genotype these mice that were used in genotypic selection to see how much of the surrounding genotype was also homozygous.

This particular aspect of the study warrants a more complete discussion. This testing involved genotyping F_3 animals from 37 to 60 cM on Chromosome 4. Due to availability, I was forced to use animals for this genotypic selection that were the progeny of genotypically selected F_2 animals for a distal region of Chromosome 4. At least one of the parents of the F_4 animals was previously selected for the same alleles from 60 to 85 cM. This region is referred to as "Tarantino selection" in Figure 3-3. Of the 161 F_4 animals, 81 were derived from an F_3 mating in which both parents had already been selected for the entire region from 37 to 85 cM.

Therefore, it is possible that a gene distal to 60 cM is the reason for the QTL. An important argument against a distal gene being responsible for the effects of this QTL is that I tested the following hypothesis. Mice derived from a mating between two "fully selected" mice had a different mean WAUC than those mice derived from one "fully selected" mouse and the other only selected from 37 to 60 cM. The results of this test were that there is not a significant difference between the two. Figure 3-4 graphically displays this test. Therefore, it is likely that the gene or genes responsible for this QTLs effects are located in the specific region examined here. However, it is possible that

many of the 101 animals derived from non-fully selected matings were homozygous throughout the distal part of Chromosome 4 just by chance.



	Increasing	Decreasing
Mean WAUC	3.49	1.63
n	37	43

	Increasing	Decreasing
Mean WAUC	3.52	1.41
n	52	29

Figure 3-4
The results of the test of the hypothesis that those mice derived from two "fully selected" parents (right) would have a different mean WAUC than those derived from one "fully selected" parent (left). There were no significant differences between the double-fully selected increasing mice and the single-fully selected mice ($p = 0.98$) or between the same categories of decreasing mice ($p = 0.68$).

Similarly to *Ssdh*-1, *Bis1*, or beta-carboline-induced seizures gene 1, is located distal to the selected region, at 80 cM. Beta-carbolines belong to the group of benzodiazepine-receptor ligands that increase neural activity. For example, methyl-beta-carboline-3-carboxylate causes convulsions at high doses (Martin et al., 1995). This gene has been nominated due to its known involvement in seizure activity.

In addition, *Cort*, or cortistatin, lies distal to the region hereby examined, but remains a candidate gene due to the possibilities mentioned before. It is located 76.5 cM

from the centromere. Near *Cort* lies *Kcnab2*, at 78.4 cM. *Kcnab2* is a potassium voltage gated channel of the shaker-related subfamily. Another candidate gene, *Znt2*, maps to 67.2 cM, slightly closer to the region under examination. *Znt2* is a zinc transporter. Crabbe nominated each of these genes based on their putative role in neurotransmission.

Closer to the QTL confirmed in this study is located *Htr1d*, another 5-hydroxytryptamine (serotonin) receptor. *Htr1d* is located 66 cM from the centromere (MGD, 1998). Serotonin receptors claim a prominent status as candidate genes for the time being, because of serotonin's wide range of effects as a neuromodulator. Studies suggest that the *Htr1d* receptors might modulate the release of neurotransmitters such as gamma-aminobutyric acid from their presynaptic location on the terminals of striatal neurons and Purkinje cells (MGD, 1998).

Reduced plasma GABA levels have been reported in alcoholics (Coffman & Petty, 1985). A decrease in GABA-mediated neurotransmission may be responsible for symptoms of alcohol withdrawal (Buck et al., 1997). Additionally, Goldstein (1975) reported that drugs aimed at GABA or other catecholamines inhibit alcohol withdrawal seizures. Furthermore, brain levels of GABA are lower than normal during the alcohol withdrawal reaction in mice treated with alcohol (Patel & Lal, 1973). Moreover, depletion of monoamines might activate the CNS pathways so as to produce seizures (Goldstein, 1975).

Interestingly, the genes that Crabbe nominated, including *Znt2*, *Kcnab2*, *Cort*, and *Bis1*, arose from a QTL analysis of control (non-alcohol) HICs. In other words,

Crabbe's QTL was nominated through testing for withdrawal seizures without even subjecting the animals to alcohol. This raises the concern that the QTL could be responsible for differences in the *sign* used to measure alcohol withdrawal severity, while not elucidating any effect on alcohol withdrawal *per se* (Horowitz & Dudek, 1983). The fact that the strain differences in handling induced convulsions are not large enough to account for the larger strain differences in ethanol withdrawal severity lays this concern to rest (Crabbe, Young, & Kosobud, 1983; Crabbe et al., 1980). Differences in metabolism, administered dose, or general excitability cannot account for the large differences in withdrawal severity between inbred lines, simply because the withdrawal severity disparity is too much greater than any differences in other seizure measures. (Crabbe, McSwigan, & Belknap, 1985).

Goldstein also addressed the possibility that strains differ only in their general excitability. However, if C57BL/6J mice were just less excitable in general than other strains, it is likely that they would be more sensitive to sedative drugs. Experimentation has shown this not to be the case (Goldstein & Kakihana, 1974). Therefore, C57BL/6J mice can at worst be described as having a more rigid set point of excitability than other strains. Goldstein's "worst-case" explanation provides just enough reason to retain *Asp2* as a candidate gene.

On the other hand, Goldstein (1975) observed that lines selectively bred for short sleep time following a single large dose of alcohol (SS) exhibited more severe withdrawal reactions than lines bred for long sleep time (LS). Although it was hypothesized that this indicated that HICs measured only a difference in general

excitability, further experimentation proved that strain differences in withdrawal severity had at least some specificity for alcohol withdrawal. For example, injections of the convulsant agent pentylenetetrazol failed to show the same strain differences as alcohol withdrawal. Goldstein therefore proposed that the differences in withdrawal severity arose fortuitously in LS and SS lines of mice (Goldstein & Kakihana, 1975). Additionally, inbred strains of mice, particularly C57BL/6 and DBA/2, differ in their susceptibility to various types of seizures (Schlesinger & Griek, 1970). This conclusion strongly suggests that handling induced convulsions during alcohol withdrawal share some neurochemical systems with susceptibility to HICs by various other treatments (Feller et al., 1994).

Furthermore, experiments with Withdrawal Seizure Prone (WSP) lines and Withdrawal Seizure Resistant (WSR) lines have shown in a variety of ways that handling induced convulsion differences are primarily differences in ethanol withdrawal sensitivity, and not general central nervous system differences. For example, McSwigan, Crabbe, & Young (1984) showed, using a number of convulsant treatments, that WSP mice are not generally more seizure prone than WSR mice.

Another confirmatory test involved audiogenic seizure testing. On one hand, WSR and WSP lines of mice did show a difference in sensitivity to audiogenic seizures at 17, 22, and 28 days. However, testing in this experiment was performed at an age range closer to the following test.

Testing of audiogenic seizures in WSP and WSR mice revealed no sensitivity difference at 71-78 days of age. Audiogenic seizures do not show significant correlation

with acute ethanol withdrawal (Belknap et al., 1993). Finally, brain synaptosomal membrane order does not appear to be related to the genetic predisposition to develop physical dependence. This logic strongly counters the nomination of *Asp2* as a candidate gene.

While the confirmation of a QTL is a valuable finding, it is imperative to note, as Lander and Schork (1994) do, that candidate genes must be subjected to rigorous evaluation before being accepted. The primary value of the current study lies in the confirmation of a QTL, rather than the possible genes that account for the QTL's effects.

Additional Concerns

An interesting aspect of withdrawal severity as a component of alcoholism is its interaction with various other putative components. For example, the inbred strain of mouse noted for greater tolerance and preference also exhibits less severe withdrawal. One theory that accounts for this discrepancy is that a strain that adapts quickly to alcohol (C57BL/6) might adapt rapidly to its absence, thus exhibiting minimal withdrawal symptoms (Littleton, 1980). In addition, Crabbe, Young, & Kosobud (1983) found a negative correlation between the hypothermic effect of ethanol and ethanol withdrawal severity. Therefore, genetic susceptibility to severe withdrawal might be predictable through hypothermic sensitivity and tolerance in the mouse.

Another noteworthy aspect of this study is that the specific region examined here for an acute withdrawal QTL was nominated explicitly for chronic alcohol withdrawal. Due to misplaced equipment the animals underwent acute withdrawal testing. The

significant outcome might attest to the complex interactive model of alcoholism proposed by Devor (1994). However, the outcome is more easily attributable to the pleiotropic nature of genes and more so the similarity of the two methods (chronic and acute) of inducing ethanol withdrawal.

Conclusions

After genetic studies assert the influence of a particular chromosomal region, such as we have done, the task of identifying the responsible gene remains (Lander & Schork, 1994).

Three general paths exist for the future use of this research, including positional cloning, candidate gene testing, and the direct identification of homologous markers or genes in humans. The most difficult to achieve is probably positional cloning, the isolation of a gene based entirely on its chromosomal location, because mapping to as small as 10 cM does not even achieve the requisite precision. A 10 to 20 cM region, which corresponds to about 10 to 20 Megabase-pairs (Mbp), could contain as many as 500 genes (Lander and Schork, 1994). In addition, very few mouse genes have been positionally cloned to date (Buck, 1995).

Candidate gene testing lies in the nearer future. As physical maps of the mouse genome continue to fill out, more genes involved in various alcohol-related phenotypes will surface. Ultimately, these genes may be tested using knockout preparations or transgenic mice. In the mean time, those genes nominated through studies like this one are prime candidates for testing (Buck, 1995).

Finally, the identification of homologous markers or genes in humans could prove to be the most effective direction in which to take animal QTL studies. As previously mentioned, the short generation time in mice allows for experimental genetic interactions far more complex than could be studied in human families. Therefore, animal studies can help identify the genes or QTLs affecting a variety of alcohol-related traits (Buck, 1995).

This third strategy is particularly valuable because mouse genomes are considerably homologous with those of humans. Therefore, mapping QTLs and genes in mice could lead directly to regions in the human genome that contain the homologous QTL or genes affecting the trait in humans (Buck, 1995). For instance, the region in which the QTL lies in this study is highly homologous with the 9p21-p23 and 1p32-p22.1 regions in humans (Buck et al., 1997). Therefore, the homologous QTL for acute alcohol withdrawal in humans might reside in one of these locations. Thousands of human genes reside in this location, some of which could be related to alcoholism. For example, the human homolog of Grik3, GRIK3, maps to Chromosome 1p (MGD, 1998).

These human risk markers, if found, might be applicable to genetic counseling. For example, "Knowledge of an individual's status with regard to risk markers could, in turn, allow assignment of relative risk with greater precision, thereby facilitating interventions before neurotoxic levels of drinking are reached (Crabbe, in press)." Since greater hangover symptoms serve as a predictor of susceptibility to alcoholism, those individuals genetically susceptible to acute withdrawal might be counseled to avoid excessive drinking.

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